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Published in:
Anaerobe

DOI:
10.1016/j.anaerobe.2017.07.004

Publication date:
2017

Anaerobe 48 (2017) 94–97

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical microbiology

A multi-center ring trial for the identification of anaerobic bacteria using MALDI-TOF MS

A.C.M. Veloo [a, *], H. Jean-Pierre [b, c], U.S. Justesen [d], T. Morris [e], E. Urban [f], I. Wybo [g], H.N. Shah [h], A.W. Friedrich [a], on behalf of the ENRIA workgroup, T. Morris [i], H.N. Shah [j], H. Jean-Pierre [k], U.S. Justesen [l], E. Nagy [m], E. Urban [m], M. Kostrzewa [n], A. Veloo [o], A.W. Friedrich [o]

[a] Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, The Netherlands
[b] Centre Hospitalier Universitaire de Montpellier, Hôpital Arnaud de Villeneuve, Laboratoire de Bactériologie, 371 Avenue du Doyen Gaston Giraud, 34295 Montpellier Cedex 5, France
[c] Université Montpellier 1, UMR5119 ECOSYM, Equipe Pathogènes Hydriques Santé Environnements, UMR 5569 Hydrosciences, UFR Pharmacie, 15 avenue Charles Flahault, 34093 Montpellier Cedex 5, France
[d] Department of Clinical Microbiology, Odense University Hospital, Odense, Denmark
[e] UK Anaerobe Reference Unit, Public Health Wales Microbiology, Cardiff, UK
[f] Institute of Clinical Microbiology, University of Szeged, Hungary
[g] Department of Microbiology and Infection Control, Universitair Ziekenhuis Brussel, Brussels, Belgium
[h] Department of Natural Sciences, Middlesex University, London NW4 4BT, UK
[i] Cardiff, Wales, UK
[j] London, England, UK
[k] Montpellier, France
[l] Odense, Denmark
[m] Szeged, Hungary
[n] Bremen, Germany
[o] Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 20 June 2017
Received in revised form
20 July 2017
Accepted 21 July 2017
Available online 7 August 2017

Handling Editor: Lyudmila Boyanova

Keywords:
Multi-center ring trial
Anaerobic bacteria
MALDI-TOF MS

ABSTRACT

Inter-laboratory reproducibility of Matrix Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF MS) of anaerobic bacteria has not been shown before. Therefore, ten anonymized anaerobic strains were sent to seven participating laboratories, an initiative of the European Network for the Rapid Identification of Anaerobes (ENRIA). On arrival the strains were cultured and identified using MALDI-TOF MS. The spectra derived were compared with two different Biotyper MALDI-TOF MS databases, the db5627 and the db6903. The results obtained using the db5627 shows a reasonable variation between the different laboratories. However, when a more optimized database is used, the variation is less pronounced. In this study we show that an optimized database not only results in a higher number of strains which can be identified using MALDI-TOF MS, but also corrects for differences in performance between laboratories.

1. Introduction

Several studies have been performed in which the use of Matrix Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has been validated for the identification of anaerobic bacteria [1,3,4,6]. The main conclusion is that identification obtained with MALDI-TOF MS is superior to phenotypic methods regarding the reliability of the identification [1,4]. Compared with phenotypic methods, it is much faster and cheaper, once the equipment is purchased [2]. Since only a minute sample is required for analysis, MALDI-TOF MS is particularly useful for identification of several fastidious and poorly growing species. Furthermore, studies have shown that the identification of anaerobic bacteria improves when the database of the MALDI-TOF MS is

* Corresponding author. Hanzeplein 1, 9713 GZ Groningen, The Netherlands..
E-mail address: a.c.m.veloo@umcg.nl (A.C.M. Veloo).

http://dx.doi.org/10.1016/j.anaerobe.2017.07.004

supplemented with reference spectra of species that are missing in the database or are underrepresented (<5 reference spectra) [12,14]. The pre-analytical factors which are required to obtain a good quality spectrum of anaerobic bacteria have also been assessed [6,10,13]. Several of these studies have been performed within the European Network for the Rapid Identification of Anaerobes (ENRIA). The main goal of this network is the optimization of the MALDI-TOF MS database for the identification of anaerobic bacteria, which also includes intra- and inter-laboratory reproducibility and comparability of MALDI-TOF MS data obtained by different laboratories.

In this study we report the results of a multi-center ring trial in which ten unknown anaerobic bacteria were identified by the core laboratories participating within the ENRIA project.

2. Material and methods

2.1. Bacterial strains

A set of 10 different anaerobic species was chosen. Each species had its own phenotypic characteristics which may influence the quality of the spectrum, e.g. colony morphology, sensitivity to oxygen and presence or absence of a reference spectrum, in the MALDI-TOF MS database. The species and their characteristics are shown in Table 1. Strain were cultured on Brucella Blood Agar (BBA, Mediaproducts, Groningen, The Netherlands) supplemented with hemin and vitamin K and incubated at 37 °C in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% H_2) for 48 h. Strains were sub cultured at least twice to ensure their viability during transport. Strains were sent to the participating laboratories using a transport medium suitable for anaerobic bacteria, Transwab® Amies charchoal medium (MWE, Wiltshire, England). The transport time was kept as short as possible. Besides viable strains, an ethanol suspension of the strain was included which can be used to perform the MALDI-TOF MS analyses if the strains failed to grow upon arrival. The participating laboratories were Center Hospitalier Universitaire de Montpellier in France, Odense University Hospital in Denmark, UK Anaerobe Reference Unit Public Health Wales Microbiology in the UK, University of Szeged in Hungary, Proteomics Research Unit of the Public Health of England in London and the Universitair Ziekenhuis Brussel in Belgium. For our own laboratory, the University Medical Center Groningen (UMCG) in The Netherlands, the transport was mimicked and the measurements were performed by a technician from the bacteriological diagnostics department.

2.2. MALDI-TOF MS analyses

MALDI-TOF MS analyses were performed as described previously [12]. Briefly, for the direct spotting method, bacterial cells were transferred to a stainless-steel target using a toothpick. After drying at ambient temperature the cells were covered with 1 μl matrix HCCA (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoro-acetic acid). An on target extraction was performed by adding 1 μl of 70% formic acid prior to the matrix. This was left to dry at ambient temperature before 1 μl of matrix HCCA was added. A full extraction was performed by suspending a 1 μl loop full of bacteria in 300 μl of distilled water. After a homogeneous suspension was obtained, 900 μl absolute ethanol was added. The bacterial suspension was centrifuged for 2 min at 13,000×*g* and the supernatant was decanted. The centrifugation step was repeated once and the remaining supernatant was carefully removed by pipetting. The pellet was dissolved in 30 μl 70% formic acid and an equal amount of acetonitrile. The suspension was centrifuged and 1 μl of the supernatant was placed on the target. Immediately after drying at ambient temperature, 1 μl of matrix HCCA was added.

Measurement were performed using the own Microflex (Bruker Daltonik GmbH, Bremen, Germany), by each participating laboratory. Prior to the measurements the MALDI-TOF MS system was calibrated using the Bacterial Test Standard (BTS, Bruker Daltonik, Germany). The obtained spectra were sent to the UMCG for further analyses.

2.3. Data analyses

All spectra received from the participating laboratories were compared with the db5627 Bruker database, which was current at the time the multi-center trial was performed in 2015, supplemented with in-house made reference spectra [13] of species which were not represented in the MALDI-TOF MS database. Two years later the same spectra were compared with the 6903 db, the current database at the time of publication.

The obtained log scores were interpreted as advised by the manufacturer. A log score of ≥2 as an identification with high confidence, a log score ≥1.7 but <2 as an identification with low confidence and log score <1.7 as no reliable identification.

For each strain identified with a log score ≥2 a participating laboratory was awarded one point. Strains which were identified with a log score ≥1.7 but <2, half a point and for strains with no reliable identification no points were received.

3. Results

The awarded points for each laboratory are summarized in Tables 2 and 3. The identifications obtained with db5627 show that the awarded points for the direct spotting and on target extraction differ between the participating laboratories from 4 to 7.5. For the full extraction less difference between the points was observed [7–8], expect for laboratory 5 which earned 5.5 points using this method. The highest total number of points was obtained by

Table 1
The anaerobic species which were used to perform the multi-center trial and their characteristics which might influence the quality of the spectrum.

Species	Characteristics
Bacteroides fragilis	None
Actinomyces israelii	Dry colonies influencing the quality of spotting
Anaerococcus murdochii	No reference spectrum present in the MALDI-TOF MS db5627
Campylobacter ureolyticus	Tiny pitting colonies influencing the quality of spotting
Solobacterium moorei	None
Robinsoniella peoriensis	No reference spectrum present in the MALDI-TOF MS db5627
Peptoniphilus coxii	No reference spectrum present in the MALDI-TOF MS db5627
Fusobacterium nucleatum	Cells get easily damaged by oxygen
Veillonella parvula	None
Cutibacterium (Propionibacterium) acnes	Thick cell wall which can hamper the quality of the spectrum

Table 2
The obtained points for each laboratory using Bruker db5627.

	Number of points						
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
Direct spotting							
Log score ≥2	3	6	4	4	3	2	6
Log score ≥1,7 and <2	2.5	1	2.5	1	1	2.5	1.5
Total	5.5	7	6.5	5	4	4.5	7.5
On target extraction							
Log score ≥2	3	5	6	4	3	5	6
Log score ≥1,7 and <2	2.5	0.5	1.5	1	2	1	0.5
Total	5.5	5.5	7.5	5	5	6	6.5
Full extraction							
Log score ≥2	8	8	7	6	4	8	7
Log score ≥1,7 and <2	0	0	0.5	1	1.5	0	0
Total	8	8	7.5	7	5.5	8	7
Total	19	20.5	21.5	17	14.5	18.5	21
Average	6.3	6.8	7.2	5.7	4.8	6.2	7.0

laboratory 3, followed by laboratory 7 and 2. The awarded points were 21.5, 21 and 20.5 respectively.

The obtained points for the direct spotting and on target extraction differs between 6.5 and 8.5, when using the db6903. The difference between the laboratories became less when the full extraction method is used. The awarded points differed between 7 and 8.5. Again laboratory 3 obtained the highest number of points (25.5), but this time followed by laboratories 1, 2 and 4, which obtained 24 points each.

All laboratories were able to identify *Bacteroides fragilis* with high confidence and not *Campylobacter ureolyticus*, regardless the method used or the database (Table 4). For the other species used in this study the laboratories performed better after the database was updated. For example, *Peptoniphilus coxii*, *Robinsoniella peoriensis*, *Fusobacterium nucleatum* and *Cutibacterium* (formerly: *Propionibacterium*) *acnes* was identified with high confidence using the on target extraction method and full extraction by all laboratories. Before the update several laboratories obtained a log score <2 for these species.

4. Discussion

The quality and reliability of a MALDI-TOF MS identification depends on several factors. Among them are; the growth phase of the bacterium, the amount of bacterial cells spotted on the target, the type of colony, whether an extraction method is used and, for anaerobic bacteria, the time the cells are exposed to oxygen. Since the basis of the method is 'pattern matching of main spectral profiles (MSPs)', the inclusion of a reference spectrum in the database is necessary for successful identification [5,7,8,12].

Table 3
The obtained points for each laboratory using Bruker db6903.

	Number of points						
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
Direct spotting							
Log score ≥2	6	8	8	8	5	7	8
Log score ≥1,7 and <2	1.5	0	0.5	0.5	1,5	0	0.5
Total	7.5	8	8.5	8.5	6.5	7	8.5
On target extraction							
Log score ≥2	8	8	8	8	7	6	7
Log score ≥1,7 and <2	0	0	0.5	0.5	0.5	0.5	0
Total	8	8	8.5	8.5	7.5	6.5	7
Full extraction							
Log score ≥2	8	8	8	7	6	8	7
Log score ≥1,7 and <2	0.5	0	0.5	0	1	0	0
Total	8.5	8	8.5	7	7	8	7
Total	24	24	25.5	24	21	21.5	22.5
Average	8	8	8.5	8	7	7.2	7.5

Table 4
Performance of the laboratories for each strain.

Strain	direct spotting number of laboratories							on target extraction number of laboratories							full extraction number of laboratories						
	Log score							Log score							Log score						
	<1.7		≥1.7 - <2		≥2			<1.7		≥1.7 - <2		≥2			<1.7		≥1.7 - <2		≥2		
	A[a]	B[b]	A	B	A	B		A	B	A	B	A	B		A	B	A	B	A	B	
Veillonella parvula	0	0	5	2	2	5		0	0	4	1	3	6		0	0	2	1	5	6	
Solobacterium moorei	0	0	2	0	5	7		1	1	0	0	6	6		0	0	1	0	6	7	
Bacteroides fragilis	0	0	0	0	7	7		0	0	0	0	7	7		0	0	0	0	7	7	
Actinomyces israelii	5	3	2	4	0	0		4	4	3	3	0	0		7	5	0	2	0	0	
Anaerococcus murdochii	1	1	5	0	1	6		4	2	3	0	0	5		2	1	0	1	5	5	
Campylobacter ureolyticus	7	7	0	0	0	0		7	7	0	0	0	0		7	7	0	0	0	0	
Peptoniphilus coxii	2	0	2	1	3	6		1	0	3	0	3	7		0	0	0	0	7	7	
Robinsoniella peoriensis	2	0	4	0	1	7		3	0	2	0	2	7		0	0	2	0	5	7	
Fusobacterium nucleatum	0	0	2	0	5	7		0	0	1	0	6	7		0	0	0	0	7	7	
Cutibacterium (Propionibacterium) acnes	1	0	2	2	4	5		0	0	2	0	5	7		0	0	1	0	6	7	

[a] Bruker db5627.
[b] Bruker db6903.

The fact that none of the participating laboratories was able to obtain a good spectrum of *C. ureolyticus*, emphasizes again that the nature of colony plays an important role for successful identification [12]. *C. ureolyticus* forms tiny pitting colonies which are very difficult to spot. Also none of the laboratories obtained a log score $\geq$2 for *Actinomyces israelii*, which is due to its very dry colonies difficult to spot. Species which were not represented in the db5627 (*Anaerococcus murdochii*, *P. coxii* and *R. peoriensis*) could not be identified by the participating laboratories, but could identified by the UMCG using homemade MSPs. The fact that these strains yielded a no reliable identification did not influence the quality of the spectra.

This study was performed in a "real life" situation in which each laboratory used its own culture media and MALDI-TOF MS system with the same settings which are used for their normal diagnostic routine. The identification obtained with MALDI-TOF MS is independent of the culture media used [11]. Evaluation of the settings of the MALDI-TOF MS system showed that the differences between the laboratories were minor (data not shown). The variation in the obtained points using db5627 can therefore be explained only by how good the spotting of the bacterial strains was performed or by the fact that the system was not in optimal condition such as a deteriorating laser or an inefficient soiled detector.

The results of this study indicate that the quality of identification is less influenced by the factors mentioned above, when the database is optimized. Previous studies have shown that more anaerobic clinical isolates are identified when more reference spectra are added to the database, including a higher log score for a part of the tested strains [13,14]. These findings are in line with our observations. Adding more reference spectra to the database not only covers the intra-species variation, but also the differences between laboratories in performing MALDI-TOF MS.

In summary, ten anonymized clinical and well characterized anaerobic isolates were identified by seven different laboratories using MALDI-TOF MS, using two different MALDI-TOF MS databases, db5627 and db6903. The performance of the laboratories differed more when a database was used containing less reference spectra of anaerobic bacteria than when using an optimized database. This emphasizes the importance of having sufficient number of representative reference spectra for anaerobic bacteria in the MALDI-TOF MS database.

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